REMARKS

Claims 7-10 and 69-71 are pending in this application. The specification was objected to as lacking sequence identifiers. Claim 8 was objected to as being a dependent claim that fails to further limit the subject matter of the previous claim. Claims 7-10 and 69-71 were rejected under 35 U.S.C. § 112, first paragraph. Each of these matters is addressed in turn below.

Objections to the specification

Sequence identifiers at pages 18 and 377 were previously added in the amendment filed July 11, 2008. Accordingly, the objection is moot.

Objections to the claims

Claim 8 was objected to as being a dependent claim that fails to further limit the subject matter of the previous claim. Claim 8 has been cancelled, and this objection may be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 7-10 and 69-71 were rejected as failing to comply with the written description requirement. The Office states "the specification only supports decreased FADS2 activity that consequently inhibits gamma-secretase activity and thereby inhibits Abeta-42 formation." The Office argues that the term "modulating" embraces compounds that activate gamma-secretase activity. The Office cites Marlow et al. (BBRC 305:502 (2003)) as teaching that increased expression of gamma-secretase component proteins increases gamma-secretase activity, resulting in increased production of Abeta-42. The Office also cites Takahashi et al. (J Biol Chem 278:18664 (2003)) as teaching that compounds that reduce the generation of Abeta-42 do so by inhibiting

gamma-secretase activity. The Office then concludes that activators of gamma-secretase activity could not reduce Abeta-42 formation.

As an initial matter, Applicants have submitted new claims 72-76 that recite "inhibiting gamma secretase activity." These claims conform to the Office's interpretation of what is supported by the current disclosure, and, therefore, allowance of these claims is respectfully requested.

Regarding the written description requirement, 35 U.S.C. § 112, first paragraph, requires that "an applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention, and that the invention, in that context, is whatever is now claimed." (M.P.E.P. § 2163.02). In performing this analysis, all of the claim limitations must be taken into account, and, when considered as a whole, the present claims meet this standard.

The remaining claims cover a method of identifying compounds that have three properties: binding to the FADS2 complex, modulating gamma secretase, and reducing Abeta-42 formation. Applicant submits that the Office has inadvertently misinterpreted the claims to cover identifying a compound that has any possible effect on gamma secretase. The method of claim 7, however, identifies compounds that are "capable of modulating gamma secretase activity **to reduce Abeta-42 formation**," i.e., the identified compound must **both** modulate gamma secretase **and** reduce Abeta-42 formation.

The term "modulating the activity of gamma secretase" is defined at paragraph 80, page 8, of the specification as published. This definition sets forth that "modulates," in the context of the present application, means either that the gamma secretase activity is "reduced in that less or no product is formed or that the respective enzyme produces a different product (in the case of gamma secretase e.g. Abeta-40 instead of Abeta-42) or that the relative quantities of the products are different (in the case of gamma secretase e.g. more Abeta-40 than Abeta-42)." The term does not include activation of gamma secretase to produce more Abeta-42. Because Applicants have defined the term

"modulating" in the specification, the claim should be interpreted consistent with this definition (M.P.E.P. § 2111.01 (IV)).

This definition of "modulating" is consistent with later observations that compounds that interact with gamma-secretase reduce Abeta-42 formation without inhibiting the overall activity of gamma-secretase. For example, Czirr et al. (J Biol. Chem. 283:17049 (2008)) describes the substance sulindac sulfide as a gamma-secretase modulator which both causes the reduction of Abeta-42 peptide formation and the increase of Abeta-38 peptide formation, i.e., the modulation of Abeta peptide formation (see e.g., the abstract and disclosure on page 17052, right column; "Confirming our sandwich immunoassay and mass spectrometry results, treatment with sulindac sulfide **reduced Abeta-42 and increased Abeta-38 levels** in PS1-WT cells," (emphasis added)).

These results contradict the Office's assertion that a reduction of Abeta-42 peptide formation is necessarily connected with the inhibition of gamma-secretase. As evidenced by the results of Czirr et al., a substance can mediate the production of different Abeta-peptide cleavage products, namely Abeta-42 and Abeta-38, respectively, and thus act as a gamma-secretase modulator as defined by the present invention.

The teachings of Marlow et al. and Takahashi do not contradict the teachings of the specification and are thus not relevant to the patentability of the present claims. Marlow et al. only teaches that certain activators of gamma secretase activity were found to increase Abeta-42 production. Such activators of gamma secretase would not be covered by the current claims, as they do not meet the limitation of reducing Abeta-42 formation. Accordingly, the reference does not call into question Applicant's possession of the invention. Takahashi teaches that certain inhibitors of gamma secretase decrease Abeta-42 formation. Takahashi does not teach that overall inhibition is the only mechanism for reducing Abeta-42. Thus, this reference also does not call into question Applicant's possession. The teachings of Takahashi are actually consistent with the

claims, as inhibitors of gamma secretase to reduce Abeta-42 formation are a type of modulation.

Finally, the present claims are directed to a screening method. One skilled in the art at the time of filing would understand how to perform each of the claimed steps and would understand whether an identified compound possessed all of the claimed properties. Thus, the claims as written are fully supported by the specification, and the rejection should be withdrawn.

CONCLUSION

Applicants submit that the claims are in condition for allowance, and such action is respectfully requested.

If there are any other charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 10/9/07

Kristina Bicker-Brady, Ph.D.
Reg. No. 39,109

J. Cooper McDonald, Ph.D.
Reg. No. 52,011

Clark & Elbing LLP
101 Federal Street
Boston, MA 02110
Telephone: 617-428-0200
Facsimile: 617-428-7045

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 25, pp. 17049–17054, June 20, 2008

Independent Generation of Aβ42 and Aβ38 Peptide Species by γ-Secretase*

Received for publication, October 15, 2007, and in revised form, April 16, 2008 Published, JBC Papers in Press, April 21, 2008, DOI 10.1074/jbc.M802912200

Eva Czirr[‡], **Barbara A. Cottrell**[§], **Stefanie Leuchtenberger**[‡], **Thomas Kukar**[¶], **Thomas B. Ladd**[¶], **Hermann Esselmann**[‖], **Sabine Paul**[‖], **Robert Schubenel**[‡‡], **Justin W. Torpey**[§§], **Claus U. Pietrzik**[¶¶], **Todd E. Golde**[¶], **Jens Wiltfang**[‖], **Karlheinz Baumann**[‡‡], **Edward H. Koo**[§], **and Sascha Weggen**[‡1]

From the [‡]Molecular Neuropathology Group, Department of Neuropathology, Heinrich Heine-University, D-40225 Duesseldorf, Germany, [§]Department of Neurosciences and [§§]Biomolecular Mass Spectrometry Facility, University of California San Diego, La Jolla, California 92093, [¶¶]Molecular Neurodegeneration Group, Institute of Physiological Chemistry and Pathobiochemistry, Johannes Gutenberg University, D-55128 Mainz, Germany, [‡‡]Pharmaceuticals Division, Preclinical Research CNS, F. Hoffmann-La Roche Ltd., CH-4070 Basel, Switzerland, [¶]Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, Florida 32224, and [‖]Department of Psychiatry and Psychotherapy, Rhine State Hospital, University of Duisburg-Essen, D-45147 Essen, Germany

Proteolytic processing of the amyloid precursor protein by β- and γ-secretase generates the amyloid-β (Aβ) peptides, which are principal drug targets in Alzheimer disease therapeutics. γ-Secretase has imprecise cleavage specificity and generates the most abundant Aβ40 and Aβ42 species together with longer and shorter peptides such as Aβ38. Several mechanisms could explain the production of multiple Aβ peptides by γ-secretase, including sequential processing of longer into shorter Aβ peptides. A novel class of γ-secretase modulators (GSMs) that includes some non-steroidal anti-inflammatory drugs has been shown to selectively lower Aβ42 levels without a change in Aβ40 levels. A signature of GSMs is the concomitant increase in shorter Aβ peptides, such as Aβ38, leading to the suggestion that generation of Aβ42 and Aβ38 peptide species by γ-secretase is coordinately regulated. However, no evidence for or against such a precursor-product relationship has been provided. We have previously shown that stable overexpression of aggressive presenilin-1 (PS1) mutations associated with early-onset familial Alzheimer disease attenuated the cellular response to GSMs, resulting in greatly diminished Aβ42 reductions as compared with wild type PS1. We have now used this model system to investigate whether Aβ38 production would be similarly affected indicating coupled generation of Aβ42 and Aβ38 peptides. Surprisingly, treatment with the GSM sulindac sulfide increased Aβ38 production to similar levels in four different PS1 mutant cell lines as compared with wild type PS1 cells. This was confirmed with the structurally divergent GSMs ibuprofen and indomethacin. Mass spectrometry analysis and high resolution urea gel electrophoresis further demonstrated that sulindac sulfide did not induce detectable compensatory changes in levels of other Aβ peptide species. These data provide evidence that Aβ42 and Aβ38 species can be independently generated by γ-secretase and argue against a precursor-product relationship between these peptides.

A variety of therapeutic strategies in clinical development for Alzheimer disease (AD),[2] the most common neurodegenerative disorder, target the amyloid-β (Aβ) peptides that are generated through proteolytic processing of the transmembrane amyloid precursor protein (APP) (1). In the Aβ-producing pathway, APP is cleaved by two aspartyl proteases, first by β-secretase within its ectodomain and subsequently by γ-secretase, which cleaves APP within its transmembrane domain (TMD) (2). γ-Secretase is a multiprotein complex with the presenilin (PS) proteins at its enzymatic core (2). Because of its imprecise cleavage specificity, γ-secretase generates Aβ peptides of variable length at the carboxyl terminus, with the highly amyloidogenic Aβ42 isoform thought to be the key pathogenic species (3). A central role of Aβ42 developed largely from genetic research demonstrating that mutations in the APP and PS genes associated with early-onset familial AD (FAD) invariably increase the Aβ42/Aβ40 ratio in primary fibroblasts and plasma of affected individuals, in transfected cells, and in transgenic animals (3).

In contrast to pan γ-secretase inhibitors that prevent all γ-secretase-mediated cleavage events in APP and other substrates, γ-secretase modulators (GSMs) have been shown to selectively lower production of the Aβ42 species without affecting other γ-secretase substrates (3–8). GSMs such as sulindac sulfide and ibuprofen were first discovered in the class of non-steroidal anti-inflammatory drugs (7), and recent derivatives have shown promise as therapeutic agents in AD animal models and clinical trials (9, 10). Several characteristics indicate that GSMs act directly on the γ-secretase complex or its substrate APP, including their activity in cell-free γ-secretase assays (4–6, 11, 12), their ability to affect conformation of presenilin-1 (PS1) (13), and the observation that overexpression of FAD PS1 mutations altered the cellular response to GSMs, resulting in enhanced or diminished Aβ42 reductions (12, 14).

* This work was supported by Emmy Noether Phase II Grant WE 2561/1–3 from the Deutsche Forschungsgemeinschaft (to S. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed. Tel.: 49-211-8104506; Fax: 49-211-8104577; E-mail: sweggen@uni-duesseldorf.de.

[2] The abbreviations used are: AD, Alzheimer disease; Aβ, amyloid β-peptide; ANOVA, analysis of variance; APP, amyloid precursor protein; FAD, early-onset familial AD; CHO, Chinese hamster ovary; GSM, γ-secretase modulator; SS, sulindac sulfide; PS, presenilin; TMD, transmembrane domain; WT, wild type; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight mass spectrometry; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

In addition, mass spectrometry analysis has shown that Aβ42-lowering GSMs induced a concomitant increase in shorter Aβ species, primarily Aβ38 (5, 7). Conversely, inverse GSMs such as fenofibrate selectively increased Aβ42 production with a concomitant decrease in Aβ38 and other shorter Aβ species (15). Interestingly, certain pan γ-secretase inhibitors elevated Aβ42 levels at subinhibitory concentrations, and this also correlated with a decrease in Aβ38 levels (16). Finally, mutations in a *GXXXG* motif in the APP TMD decreased Aβ42 generation and increased Aβ38 levels from the mutant substrate (17). Taken together, these findings strongly indicated coordinated production of Aβ42 and shorter species such as Aβ38 by γ-secretase, but no confirmation for or against interdependence between these peptides has been provided so far. Importantly, this issue is of significance not only for the mode of action of GSMs but also to understand the molecular mechanism of Aβ generation by γ-secretase. In this respect, it has been proposed that Aβ peptides are generated by sequential cleavage of longer into shorter peptide species (16–21). Further, FAD PS1 mutations might lower the catalytic activity of γ-secretase, thereby reducing the turnover of Aβ42 into shorter species (18, 20, 22, 23). We have recently shown that several FAD PS1 mutations, characterized by their aggressive nature with disease onset in the second to fourth decade in life, rendered cells non-responsive to the ability of GSMs to lower Aβ42 (14). Now, we have used this tissue culture model to investigate a potential precursor-product relationship between Aβ42 and Aβ38 peptide species. Unexpectedly, treatment with structurally divergent GSMs increased Aβ38 production to similar levels in PS1 mutant cell lines as compared with wild type PS1 control cells despite the insensitivity of the mutants to Aβ42 reduction. These data are inconsistent with strictly coordinated cleavages and support independent generation of Aβ42 and the shorter Aβ38 peptides by γ-secretase.

EXPERIMENTAL PROCEDURES

Drugs, Antibodies, Cell Lines, and Cell Culture—The nonsteroidal anti-inflammatory drugs sulindac sulfide, ibuprofen, and indomethacin were purchased from BIOMOL (Plymouth Meeting, PA). All other chemicals were from Sigma-Aldrich. Monoclonal antibody Ab9 against amino acids 1–16 of human Aβ and the carboxyl terminus-specific Aβ antibodies BAP24, BAP15, and BAP29 have been described (24, 25). Biotinylated monoclonal antibody 6E10 recognizing amino acids 1–17 of human Aβ was purchased from Signet (Dedham, MA). Generation of Chinese hamster ovary (CHO) cells with stable coexpression of wild type APP751 and wild type PS1 or the PS1 mutations PS1-P117L, PS1-L166P, PS1-G384A, and PS1-ΔExon9 has been described previously, and comparable PS1 and APP expression in all cell lines has been demonstrated (14). All cell lines were maintained in α-minimum essential medium supplemented with 10% fetal bovine serum, 1 mM sodium-pyruvate, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin (Invitrogen).

Dose-response · Experiments and Statistical Analysis—Aβ secretion of individual cell lines after GSM treatments was compared in dose-response experiments as described (14). All cell lines intended for comparison were cultured and treated in parallel at similar cell densities. Cells were cultured in serum-containing medium and treated for 24 h with indicated concentrations of GSMs or Me_2SO vehicle. Aβ40, Aβ42, and Aβ38 levels in conditioned medium were then analyzed by sandwich immunoassay. Duplicate measurements from each drug concentration were averaged and normalized to Me_2SO control condition. These experiments were repeated five times, and results were analyzed by one-way analysis of variance (ANOVA) with Dunnett's post tests using GraphPad Prism software (San Diego, CA).

Aβ Liquid Phase Electrochemiluminescence Assay—Aβ levels were analyzed by sandwich immunoassay as described (14, 24). In brief, the biotinylated antibody 6E10 was used as capture antibody, and carboxyl terminus-specific Aβ antibodies BAP24, BAP15, and BAP29 were labeled with TAG electrochemiluminescent label (Bioveris, Gaithersburg, MD) and used for detection. Culture media were collected following conditioning for 24 h, cell debris was removed, and complete protease inhibitor mixture (Roche Diagnostics) was added. For liquid phase electrochemiluminescence assay analysis, conditioned media were incubated for 3 h with M-280 paramagnetic beads (Invitrogen) and antibodies 6E10 and BAP24-TAG (for Aβ40), BAP15-TAG (Aβ42), or BAP29-TAG (Aβ38). Electrochemiluminescence was quantified using an M-Series M8 analyzer (Bioveris).

Mass Spectrometry Analysis—Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry of Aβ peptides was performed on a 4800 MALDI-TOF-TOF (Applied Biosystems/MDS-Sceix, Foster City, CA). Aβ peptides were immunoprecipitated from conditioned medium with Ab9 antibody covalently coupled to Seize™ beads (Pierce). Peptides were eluted from the beads with 25% 0.1% trifluoroacetic acid:75% acetonitrile. Samples were mixed 1:1 with α-cyano-4-hydroxycinnamic acid matrix in methanol:acetonitrile:water (36%:56%:8%) (Agilent, Santa Clara, CA) and spotted on the MALDI target. Mass spectra were acquired from *m/z* 3500–5000 Da in reflector positive mode at 10,000 shots/spectrum using single shot protection and a delayed extraction time of 420 ns. The area of the isotope pattern (isotopic cluster area) was used as a measure of apparent relative abundance and expressed as a % of total. Results from three independent experiments were averaged for statistical analysis.

Urea Gel Electrophoresis—Cells were lysed in radioimmune precipitation buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 25 mM Tris-HCl, pH 7.6, 150 mM NaCl), and Aβ peptides from 1.25 mg of total protein were immunoprecipitated using 25 μl of Dynabeads (Dynal Biotech, Hamburg, Germany) coated with 1E8 monoclonal antibody directed against the amino terminus of Aβ (Bayer-Schering AG). Immunoprecipitated material or conditioned supernatants were separated on 10% Bicine/Tris gels containing 8 M urea (22), and Aβ peptide species were revealed by Western blotting using antibody 1E8. Synthetic Aβ peptides of different size were run in parallel in the same gel system and under the same conditions for the identification and quantification of Aβ peptides by densitometry. Immunoreactive band intensities were quantified with the Quantity One v4.1 software (Bio-Rad). All samples were run as duplicates, and each gel carried a five-step dilution

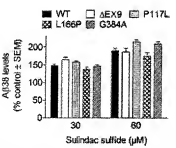

FIGURE 1. **Independent generation of Aβ42 and Aβ38 peptides by γ-secretase in cell lines expressing aggressive PS1 mutants.** CHO cells with stable co-expression of wild type APP and wild type PS1 (PS1-WT) or FAD PS1 mutants were treated with increasing concentrations of the γ-secretase modulator sulindac sulfide or Me_2SO vehicle, and Aβ38 levels in conditioned medium were quantified by sandwich immunoassay. We have previously shown that cell lines expressing the FAD mutants PS1-ΔExon9, PS1-P117L, PS1-L166P, and PS1-G384A were non-responsive to the Aβ42-lowering activity of sulindac sulfide and did not show reductions in Aβ42 levels, whereas Aβ42 levels were significantly reduced in PS1-WT control cells (14). However, Aβ38 levels were increased in a dose-dependent manner in PS1 mutant cell lines with no significant difference as compared with PS1-WT control cells (Table 1), indicating that Aβ42 and Aβ38 peptides can be generated independently by γ-secretase. Dose-response experiments ($n = 5$) were analyzed by one-way ANOVA with PS1-WT cells as control group.

series of a synthetic Aβ peptide mix. Bands were quantified relative to this dilution series. The inter- and intra-assay coefficients were below 10%. Mean values were used for subsequent calculations.

RESULTS AND DISCUSSION

We previously demonstrated that aggressive PS1 mutations associated with early onset FAD displayed a dramatic increase in the Aβ42/Aβ40 ratio (14). Further, these mutants were partially non-responsive to pan γ-secretase inhibitors *in vitro* and *in vivo*, indicating that certain AD tissue culture and animal models harboring aggressive PS1 mutations might not be suitable to assess the potency and efficacy of γ-secretase inhibitors. In the same study, we showed that these PS1 mutations were also insensitive to the Aβ42-lowering activity of GSMs (14). For these experiments, CHO cells with stable co-expression of APP and wild type PS1 (PS1-WT) or the PS1 mutants PS1-P117L, PS1-L166P, PS1-G384A, and PS1-ΔExon9 were treated with 30–60 μM of the GSM sulindac sulfide (SS), Aβ42 levels in corresponding cell culture supernatants were determined, and Aβ42 secretion of individual cell lines was assessed. All cell lines expressing mutant PS1 were either completely refractory to SS (PS1-ΔExon9, PS1-L166P) with no Aβ42 reductions or showed only minor Aβ42 reductions (10% reduction at 60 μM, PS1-P117L, PS1-G384A), whereas PS1-WT control cells displayed robust dose-dependent reductions in Aβ42 levels (40% at 60 μM) (14). We now took advantage of this culture system to investigate a potential precursor-product relationship between Aβ42 and Aβ38 peptides by measuring Aβ38 levels in culture supernatants from the same experiments. If Aβ38 production by γ-secretase were strictly, inversely coupled to Aβ42 production or were dependent on Aβ42 turnover, then treatment of PS1 mutant cell lines with GSMs should also result in greatly diminished Aβ38 elevations as compared with cells expressing PS1-WT. However, we found that sulindac sulfide treatment robustly increased Aβ38 levels in a dose-dependent manner

TABLE 1

Aβ38 levels are similarly increased in cell lines expressing wild type or mutant PS1 after treatment with the γ-secretase modulator sulindac sulfide

Dose-response experiments were performed as described under "Experimental Procedures," and analyzed by one-way ANOVA with PS1-WT cells as control group. $n = 5$.

	Sulindac sulfide	
Cell line	Aβ38 levels (% control ± S.E.)	
	30 μM	60 μM
PS1-WT	147.88 ± 3.37	190.44 ± 5.65
PS1-ΔExon9	164.01 ± 7.49	186.59 ± 10.81
PS1-P117L	157.00 ± 3.55	215.01 ± 5.55
PS1-L166P	137.77 ± 5.62	175.07 ± 9.19
PS1-G384A	146.37 ± 4.84	209.264 ± 6.56

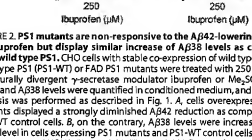

FIGURE 2. **PS1 mutants are non-responsive to the Aβ42-lowering activity of ibuprofen but display similar increase of Aβ38 levels as compared with wild type PS1.** CHO cells with stable co-expression of wild type APP and wild type PS1 (PS1-WT) or FAD PS1 mutants were treated with 250 μM of the structurally divergent γ-secretase modulator ibuprofen or Me_2SO vehicle. Aβ42 and Aβ38 levels were quantified in conditioned medium, and statistical analysis was performed as described in Fig. 1. *A*, cells overexpressing FAD mutants displayed a strongly diminished Aβ42 reduction as compared with PS1-WT control cells. *B*, on the contrary, Aβ38 levels were increased to the same level in cells expressing PS1 mutants and PS1-WT control cells (Table 2). $n = 5$; one-way ANOVA, **, $p < 0.01$, ***, $p < 0.001$ Dunnett's post tests.

from all PS1 mutant cell lines without any significant difference as compared with PS1-WT control cells (Fig. 1 and Table 1). To exclude a compound-specific effect, we repeated these experiments with the structurally divergent GSM ibuprofen, which belongs to the aryl propionic acid class of non-steroidal anti-inflammatory drugs (Fig. 2). Cell lines expressing PS1-WT or mutant PS1 were treated with 250 μM ibuprofen, and Aβ40, Aβ42, and Aβ38 levels in culture supernatants were measured and compared by one-way ANOVA. At this concentration, ibuprofen did not cause toxicity in CHO cells (7) and no significant reductions in Aβ40 levels were observed (data not shown). In PS1-WT control cells, ibuprofen induced a strong reduction in Aβ42 levels whereas all cell lines expressing PS1 mutants displayed a significantly attenuated response (Fig. 2*A* and Table 2). In contrast, comparable with our findings with sulindac sulfide, Aβ38 levels were elevated by ibuprofen treatment in PS1 mutant cell lines indistinguishable to PS1-WT control cells (Fig. 2*B* and Table 2). This was further confirmed with the GSM indomethacin (data not shown).

To examine the possibility that PS1 mutants would cause overall alterations in the pattern of Aβ peptides or that GSM treatment would induce compensatory changes in other species not detectable with our carboxyl terminus-specific Aβ antibodies, we analyzed the full spectrum of Aβ peptides

secreted by PS1-WT control cells or cells expressing PS1-L166P or PS1-ΔExon9 by mass spectrometry. Cells were treated with 60 μM SS, and tissue culture supernatants were immunoprecipitated with antibody Ab9 recognizing amino acids 1–16 of the human Aβ sequence. The immunoprecipitated material was then analyzed by MALDI-TOF-TOF. To determine the relative abundance of individual Aβ peptides, the area of the isotopic cluster for each peptide was analyzed (Fig. 3). We observed that this tended to be more sensitive than measuring peak height alone (data not shown). The profiles of Aβ peptides produced by cells expressing PS1 mutants have not been examined in depth previously. Interestingly, although the full range of Aβ peptides from Aβ31–33 to Aβ1–40 and Aβ42 was produced, we observed that Aβ34 levels appeared slightly higher in PS1 mutant cells as compared with PS1-WT control cells (Fig. 3A). Following SS treatment, the MALDI-TOF results also confirmed our sandwich immunoassay data, namely, cells expressing PS1 mutants displayed an attenuated response to SS with diminished Aβ42 reductions as compared with PS1-WT control cells, whereas the levels of Aβ38 were increased in all cell lines. More importantly, no substantial additional changes in the pattern of Aβ peptides were observed after SS treatment in PS1 mutant cells or PS1-WT control cells (Fig. 3). However, it remains plausible that Aβ38 peptides could be generated by trimming of other longer species such as Aβ45/Aβ46 in PS1 mutant cells. In support of this possibility, replacement of the Aβ40 and Aβ42 cleavage sites in the APP TMD by tryptophan mutagenesis abolished Aβ40 and Aβ42 secretion and caused accumulation of longer Aβ peptides in cell lysates but still allowed secretion of Aβ38 (26). The highly hydrophobic Aβ peptides longer than Aβ42 are inefficiently secreted and cannot be analyzed by mass spectrometry (19). Consequently, we used high resolution urea gel electrophoresis to examine longer Aβ species in cell lysates. In cell supernatants, we were able to detect Aβ peptides ranging from Aβ37 to Aβ40 and Aβ42 by urea gel electrophoresis as previously shown (22). Confirming our sandwich immunoassay and mass spectrometry results, treatment with sulindac sulfide reduced Aβ42 and increased Aβ38 levels in PS1-WT cells, whereas in cells expressing PS1 mutants L166P or G384A Aβ38 levels were increased despite unchanged Aβ42 levels (Fig. 4A). In corresponding cell lysates from the same experiments (Fig. 4B), we further detected minute amounts of longer Aβ species such as Aβ44, Aβ45, and Aβ46, but these peptides were not differentially affected by GSM treatment. In fact, careful quantitative analysis of several independent experiments showed that

TABLE 2

PS1 mutants are non-responsive to the Aβ42-lowering activity of ibuprofen but display similar increase of Aβ38 levels as compared to wild type PS1

Dose-response experiments were performed as described under "Experimental Procedures," and analyzed by one-way ANOVA with PS1-WT cells as control group. $n = 5$.

	Ibuprofen	
Cell line	Aβ42 levels (% control ± S.E.) 250 μM	Aβ38 levels (% control ± S.E.)
PS1-WT	62.89 ± 3.31	142.50 ± 5.31
PS1-ΔExon9	94.26 ± 4.21[a]	127.40 ± 8.52
PS1-P117L	81.37 ± 3.60[a]	139.90 ± 2.82
PS1-L166P	111.40 ± 4.90[a]	126.10 ± 4.82
PS1-G384A	88.40 ± 1.80 [a]	134.30 ± 4.44

[a] $P < 0.001$ Dunnett's post tests.
[b] $P < 0.01$.

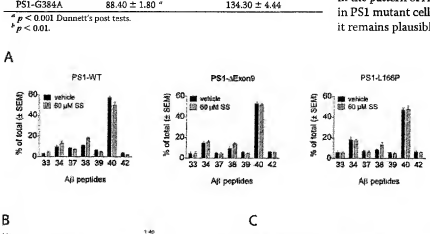

FIGURE 3. **Mass spectrometry analysis of Aβ peptide species after treatment with the γ-secretase modulator sulindac sulfide.** *A*, CHO cells with stable co-expression of wild type APP and wild type PS1 (*PS1-WT*) or FAD mutants PS1-ΔExon9 or PS1-L166P were treated with 60 μM of the γ-secretase modulator sulindac sulfide (SS) or Me_2SO vehicle. Tissue culture supernatants were immunoprecipitated with antibody Ab9 recognizing amino acids 1–16 of the human Aβ sequence and analyzed by MALDI-TOF-TOF. Peptides ranging from Aβ1–33 to Aβ1–40 and Aβ42 were detected. To determine the relative abundance of individual Aβ peptides, the area of the isotopic cluster from three independent experiments was averaged and then presented as % total of all observed signals. Confirming our results with sandwich immunoassay detection, cells expressing either PS1-ΔExon9 (*middle panel*) or PS1-L166P (*right panel*) mutants displayed an attenuated response to SS with diminished Aβ42 reductions as compared with PS1-WT control cells (*left panel*), whereas Aβ38 levels were induced in all cell lines. Aβ34 levels appeared slightly higher in PS1 mutant cell lines as compared with PS1-WT cells. However, no substantial additional changes in the pattern of Aβ peptides were observed after SS treatment in PS1 mutant cells or PS1-WT control cells, demonstrating that γ-secretase modulator treatment did not induce compensatory changes in other species not detectable by sandwich immunoassay. *B*, representative mass spectrometry spectra to PS1-WT control cells treated with vehicle (*upper panel*) or 60 μM SS (*lower panel*). Each spectrum is normalized to the tallest peak (Aβ1–40), and the percentage of Aβ1–42 relative to Aβ1–40 is shown in the expanded inserts. Y-axis is relative intensity. *C*, representative mass spectrometry spectra from PS1-L166P cells treated with vehicle (*upper panel*) or 60 μM SS (*lower panel*).

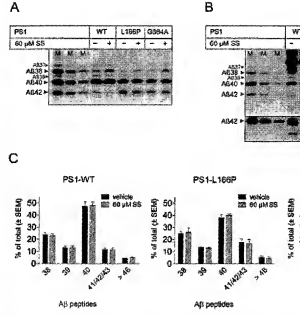

FIGURE 4. **Urea gel electrophoresis analysis of Aβ peptide species after treatment with the γ-secretase modulator sulindac sulfide.** *A*, CHO cells with stable co-expression of wild type APP and wild type PS1 (*PS1-WT*) or FAD mutants PS1-L166P or PS1-G384A were treated with 60 μM of the γ-secretase modulator sulindac sulfide (SS) or Me_2SO vehicle. Cell supernatants were separated on urea SDS-gels, and Aβ peptides were detected by Western blotting with antibody 1E8. The first three lanes (*M*) show serial dilutions of synthetic marker peptides for the identification of matching Aβ peptide species in the cell supernatants. Peptides ranging from Aβ1–37 to Aβ1–40 and Aβ1–42 were detected. In accordance with the sandwich immunoassay and mass spectrometry results, cells expressing PS1 mutants displayed diminished Aβ42 reductions in cell supernatants after SS treatment as compared with PS1-WT cells, whereas Aβ38 levels were increased in cell supernatants of both PS1-WT and PS1 mutant cells. Other detectable Aβ peptide species, Aβ37 and Aβ39, were unchanged by SS treatment. One of three representative experiments is shown. *B*, analysis of Aβ peptide species in corresponding cell lysates of the same experiment. Aβ peptides were immunoprecipitated from cell lysates with antibody 1E8, the immunoprecipitated material was separated by urea gel electrophoresis, and Aβ peptides were detected by Western blotting with antibody 1E8. In addition to the Aβ peptide species detected in cell supernatants, cell lysates contained minute amounts of peptides longer than Aβ42, such as Aβ44, Aβ45, and Aβ46. Levels of these peptides were not affected by SS treatment in either PS1-WT or PS1 mutant cells. The *lane on the right* side shows separation of a mixture of synthetic peptides ranging from Aβ41 to Aβ49. The urea gel system did not allow clear separation of Aβ41, Aβ42, and Aβ43 or peptides Aβ46, Aβ47, Aβ48, and Aβ49. The *lower panel* shows a longer exposure of the same gel for the area containing peptides >Aβ42. *C*, densitometric quantification of Aβ peptide species from three independent experiments demonstrated that the intracellular pool of all detectable Aβ peptides, including Aβ42 and Aβ38, was unchanged by SS treatment in either PS1-WT or PS1 mutant cells.

the intracellular pool of all detectable Aβ peptides, including Aβ42 and Aβ38, was not affected by GSM treatment in either PS1-WT or PS1 mutant cells (Fig. 4*C*). We have reported previously that sulindac sulfide treatment was able to lower intracellular Aβ42 levels in CHO cells with stable co-expression of APP and the PS1 mutant PS1-M146L (12). However, in the same study, we showed that the PS1-M146L mutation strongly enhances the cellular response to Aβ42-lowering GSMs as compared with PS1-WT (12). Furthermore, at the time, our analysis of intracellular Aβ species using less developed protocols for immunoprecipitation and Western blot detection was evidently close to the detection limit. Our new findings now clearly demonstrate that sulindac sulfide treatment exclusively affects the secreted pool of Aβ42 peptides. This observation in conjunction with the very low abundance of longer Aβ peptides in our cell lines argues against the possibility that the increased Aβ38 levels in PS1 mutant cells after GSM treatment can be explained by enhanced turnover of intracellular Aβ peptides longer than Aβ42. In conclusion, these data demonstrate that Aβ42 and Aβ38 peptides can be generated independently by γ-secretase and that the production of these peptides is not constrained by a stringent precursor-product relationship.

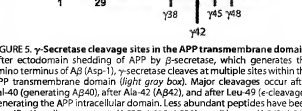

FIGURE 5. **γ-Secretase cleavage sites in the APP transmembrane domain.** After ectodomain shedding of APP by β-secretase, which generates the amino terminus of Aβ (Asp-1), γ-secretase cleaves at multiple sites within the APP transmembrane domain (*light gray box*). Major cleavages occur after Val-40 (generating Aβ40), after Ala-42 (Aβ42), and after Leu-49 (ε-cleavage), generating the APP intracellular domain. Less abundant peptides have been identified in cell supernatants (Aβ37, Aβ38, Aβ39) or cell lysates (Aβ43, Aβ45, Aβ46, Aβ48). α-Helical models of the APP TMD align the cleavage sites for Aβ40, Aβ43, Aβ46, and ε-cleavage on one surface of the helix and cleavage sites for Aβ38, Aβ42, Aβ45, and Aβ48 on the opposite surface. Cleavages may occur simultaneously and independently of each other. Alternatively, cleavages may occur sequentially at every 3–4 residues along the α-helical surface. Our data indicate that Aβ42 and Aβ38 peptides can be generated independently by γ-secretase and that the production of these peptides is not defined by a precursor-product relationship.

How do our observations conform to the current models of Aβ generation by γ-secretase? After ectodomain shedding of APP by β-secretase, the remaining membrane-bound fragment becomes a substrate for γ-secretase and is cleaved at multiple sites within its TMD (Fig. 5). The abundance of proteolytic products indicates that predominant γ-secretase cleavages occur after Val-40 (generating Aβ40), after Ala-42 (Aβ42), and after Leu-49 (ε-cleavage), the latter producing the APP intracellular domain, a cytosolic fragment with potential signaling functions (2). Less abundant peptides are generated by cleavage after various other residues and have been identified in cell supernatants (Aβ37, Aβ38, Aβ39) or cell lysates (Aβ43, Aβ45, Aβ46, Aβ48) (19). Evidence indicates that the APP TMD has α-helical conformation with 3.6 residues forming one complete turn (27, 28), which would align the cleavage sites for Aβ40, Aβ43, Aβ46, and ε-cleavage on one surface of the helix and cleavage sites for Aβ38, Aβ42, Aβ45, and Aβ48 on the opposite surface (Fig. 5). It remains to be clarified whether these cleavages occur simultaneously and independently of each other or

whether Aβ peptides are generated by sequential trimming of longer into shorter species (18, 20). If the cleavages occur independently of each other, then FAD-associated mutations in PS or APP or GSM treatment might simply result in subtle changes in substrate presentation to the active site of γ-secretase (3). In case of a γ-secretase complex containing PS1-WT, GSM treatment might favor exposure of the peptide bond at the Aβ38 cleavage site at the expense of production of the longer Aβ42 peptide. In contrast, aggressive PS1 mutations that are non-responsive to GSMs might confer drastic conformational changes on the γ-secretase that are only partially reversible by GSMs, resulting in increased Aβ38 production in the absence of corresponding Aβ42 reductions. On the other hand, several groups have provided strong evidence that Aβ peptides might be generated by sequential proteolytic cleavage, with ε-cleavage occurring first and subsequent cleavages taking place at every 3–4 residues along the α-helical surface (16, 17, 19, 21, 26). In this model, and in accordance with findings that many FAD PS mutations seemed to reduce the catalytic activity of γ-secretase (22, 23), γ-secretase complexes containing mutant PS might be more prone to release Aβ42 from the active site before further trimming to Aβ38 (18, 20). Conversely, GSMs may strengthen the substrate-enzyme interaction, thereby enhancing the turnover from Aβ42 to Aβ38. Our findings that production of Aβ42 and Aβ38 peptides can be uncoupled favors the first model of independent cleavages by γ-secretase. However, our results do not refute the concept of Aβ generation by sequential γ-secretase cleavage. Our tissue culture model using PS1 mutants and GSM treatment did only allow examining the relationship between Aβ42 and Aβ38 species. Furthermore, we cannot exclude that the presence of mutant PS1 in the γ-secretase complex somehow uncouples Aβ42 and Aβ38 production. During preparation of this report, we learned that another laboratory had also observed uncoupling of Aβ42 and Aβ38 generation after GSM treatment in cell lines expressing mutant PS1 and in an AD transgenic mouse model expressing mutant PS2 (29). Taken together, these and our findings suggest that a pure sequential cleavage model could be an oversimplification and that the mechanism of Aβ generation by γ-secretase might be even more complex than previously assumed. Moreover, it raises the possibility that selective modulators of Aβ42 production might exist that do not cause induction of shorter Aβ peptides. This is also supported by the observation that the inverse GSM celecoxib did not affect Aβ38 levels (15). As a consequence, reliance on Aβ38 elevation as more easily detectable, surrogate readout could lead to false negative results in drug screenings for Aβ42-lowering GSMs.

Acknowledgments—We thank Dr. Manfred Brockhaus (F. Hoffmann-La Roche Ltd.) for providing carboxyl terminus-specific Aβ antibodies, Drs. Dirk Beher (Amgen), Bart de Strooper, and Lutgarde Serneels (KU Leuven, Belgium) for scientific input, Elisabeth Stein for help with the figures, and Dr. Guido Reifenberger (University of Düsseldorf, Germany) for encouragement and support.

REFERENCES

1. Golde, T. E. (2006) *J. Neurochem.* **99**, 689–707
2. Wolfe, M. S. (2006) *Biochemistry* **45**, 7931–7939
3. Leuchtenberger, S., Beher, D., and Weggen, S. (2006) *Curr. Pharm. Des.* **12**, 4337–4355
4. Beher, D., Clarke, E. E., Wrigley, J. D., Martin, A. C., Nadin, A., Churcher, I., and Shearman, M. S. (2004) *J. Biol. Chem.* **279**, 43419–43426
5. Eriksen, J. L., Sagi, S. A., Smith, T. E., Weggen, S., Das, P., McLendon, D. C., Ozols, V. V., Jessing, K. W., Zavitz, K. H., Koo, E. H., and Golde, T. E. (2003) *J. Clin. Investig.* **112**, 440–449
6. Takahashi, Y., Hayashi, I., Tominari, Y., Rikimaru, K., Morohashi, Y., Kan, T., Natsugari, H., Fukuyama, T., Tomita, T., and Iwatsubo, T. (2003) *J. Biol. Chem.* **278**, 18664–18670
7. Weggen, S., Eriksen, J. L., Das, P., Sagi, S. A., Wang, R., Pietrzik, C. U., Findlay, K. A., Smith, T. E., Murphy, M. P., Bulter, T., Kang, D. E., Marquez-Sterling, N., Golde, T. E., and Koo, E. H. (2001) *Nature* **414**, 212–216
8. Weggen, S., Eriksen, J. L., Sagi, S. A., Pietrzik, C. U., Golde, T. E., and Koo, E. H. (2003) *J. Biol. Chem.* **278**, 30748–30754
9. Imbimbo, B. P., Del Giudice, E., Colavito, D., D'Arrigo, A., Dalle Carbonare, M., Villetti, G., Facchinetti, F., Volta, R., Pietrini, V., Baroc, M. F., Serneels, L., De Strooper, B., and Leon, A. (2007) *J. Pharmacol. Exp. Ther.* **323**, 822–830
10. Wilcock, G. K., Black, S. E., Haworth, J., Hendrix, S., Laughlin, M., Zavitz, K., Christensen, D., Bass, S., and Swabb, E. (2006) *Alzheimers Dement.* **2**, S81–S82
11. Fraering, P. C., Ye, W., Strub, J. M., Dolios, G., LaVoie, M. J., Ostaszewski, B. L., van Dorsselaer, A., Wang, R., Selkoe, D. J., and Wolfe, M. S. (2004) *Biochemistry* **43**, 9774–9789
12. Weggen, S., Eriksen, J. L., Sagi, S. A., Pietrzik, C. U., Ozols, V., Fauq, A., Golde, T. E., and Koo, E. H. (2003) *J. Biol. Chem.* **278**, 31831–31837
13. Lleo, A., Berezovska, O., Herl, L., Raju, S., Deng, A., Bacskai, B. J., Frosch, M. P., Irizarry, M., and Hyman, B. T. (2004) *Nat. Med.* **10**, 1065–1066
14. Czirr, E., Leuchtenberger, S., Dorner-Ciossek, C., Schneider, A., Jucker, M., Koo, E. H., Pietrzik, C. U., Baumann, K., and Weggen, S. (2007) *J. Biol. Chem.* **282**, 24504–24513
15. Kukar, T., Murphy, M. P., Eriksen, J. L., Sagi, S. A., Weggen, S., Smith, T. E., Ladd, T., Khan, M. A., Kache, R., Beard, J., Dodson, M., Merit, S., Ozols, V. V., Anastasiadis, P. Z., Das, P., Fauq, A., Koo, E. H., and Golde, T. E. (2005) *Nat. Med.* **11**, 545–550
16. Zhao, G., Tan, J., Mao, G., Cui, M. Z., and Xu, X. (2007) *J. Neurochem.* **100**, 1234–1246
17. Munter, L. M., Voigt, P., Harmeier, A., Kaden, D., Gottschalk, K. E., Weise, C., Pipkorn, R., Schaefer, M., Langosch, D., and Multhaup, G. (2007) *EMBO J.* **26**, 1702–1712
18. De Strooper, B. (2007) *EMBO Rep.* **8**, 141–146
19. Qi-Takahara, Y., Morishima-Kawashima, M., Tanimura, Y., Dolios, G., Hirotani, N., Horikoshi, Y., Kametani, F., Maeda, M., Saido, T. C., Wang, R., and Ihara, Y. (2005) *J. Neurosci.* **25**, 436–445
20. Wolfe, M. S. (2007) *EMBO Rep.* **8**, 136–140
21. Zhao, G., Cui, M. Z., Mao, G., Dong, Y., Tan, J., Sun, L., and Xu, X. (2005) *J. Biol. Chem.* **280**, 37689–37697
22. Kentmir, M., Nyabi, O., Verhamme, J., Tolia, A., Horre, K., Wiltfang, J., Esselmann, H., and De Strooper, B. (2006) *J. Neurochem.* **96**, 732–742
23. Walker, E. S., Martinez, M., Brunkan, A. L., and Goate, A. (2005) *J. Neurochem.* **92**, 294–301
24. Narlawar, R., Perez Revuelta, B. I., Baumann, K., Schubenel, R., Haass, C., Steiner, H., and Schmidt, B. (2007) *Bioorg. Med. Chem. Lett.* **17**, 176–182
25. Levites, Y., Das, P., Price, R. W., Rochette, M. J., Kostura, L. A., McGowan, E. M., Murphy, M. P., and Golde, T. E. (2006) *J. Clin. Investig.* **116**, 193–201
26. Sato, T., Tanimura, Y., Hirotani, N., Saido, T. C., Morishima-Kawashima, M., and Ihara, Y. (2005) *FEBS Lett.* **579**, 2907–2912
27. Lichtenthaler, S. F., Wang, R., Grimm, H., Uljon, S. N., Masters, C. L., and Beyreuther, K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 3053–3058
28. Wolfe, M. S., Xia, W., Moore, C. L., Leatherwood, D. D., Ostaszewski, B., Rahmati, T., Donkor, I. O., and Selkoe, D. J. (1999) *Biochemistry* **38**, 4720–4727
29. Page, R. M., Baumann, K., Tomioka, M., Perez-Revuelta, B. I., Fukumori, A., Jacobsen, H., Flohr, A., Luebbers, T., Ozmen, L., Steiner, H., and Haass, C. (2008) *J. Biol. Chem.* **283**, 677–683